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## Antioxidant Status and Sex Hormones in Women with Simple Endometrial Hyperplasia

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### Abstract

Cancer of the reproductive tract is an important source of morbidity and mortality among women worldwide. Factors affecting endometrial cancer and endometrial hyperplasia are known to be similar. Endometrial hyperplasia is abnormal proliferation of the glands and the stroma resulting in architectural and cytological modifications. Due to hormonal changes, this condition is most common among women who are nearing the menopause or have reached the menopause. Antioxidant system has a role in preventing cancer initiation and promotion. Since the carcinogenesis occurs in several stages, it is likely that the antioxidant defense depends on the type of cell and tissue. The objective of this study was to investigate whether antioxidant enzymes activities and lipid hydroperoxides concentration in patients with endometrial hyperplasia are influenced by the changes in sex hormones level (estradiol, progesterone, FSH, and LH) during the menstrual cycle and in postmenopause. The material we used consisted of blood and endometrial tissue specimens of women diagnosed with endometrial hyperplasia simplex. Patients were divided in groups depending on the phase of the menstrual cycle: follicular phase, luteal phase and postmenopause. The activities of antioxidant enzymes and the lipid hydroperoxides level were compared among the phases to test the differences and a linear regression model was used to evaluate the associations between hormone levels and antioxidant/oxidant variables. In the blood of examined patients, we observed a phase-related changes of LOOH concentrations. Significant negative correlation between FSH concentration and GR activity ( $r = -0.42$ ,  $p < 0.05$ ) and significant positive correlation between LH and

LOOH concentrations ( $r = 0.038$ ,  $p < 0.05$ ) was found. In hyperplasia simplex tissue we recorded significant phase-related changes of LOOH level as well as of AO enzyme activities. SOD and CAT had similar activity pattern, which was higher in luteal phase and in postmenopause, compared to follicular phase ( $p < 0.05$ ). GPx and GR activities did not show any statistical difference. Also, negative correlation between progesterone and GR activity ( $r = -0.036$ ,  $p < 0.05$ ) was observed. Hormonal influence on AO system is of importance in gynecological diseases etiology since they may promote cell proliferation but are also used in conservative therapy, especially for hyperplasia simplex. However, the role of ROS production as a risk factor for endometrial hyperplasia still needs to be clarified as well as the role of AO status in response to gonadotropins and sex steroids.

**Keywords:** antioxidant enzymes, lipid hydroperoxides, gonadotropins, estradiol, progesterone, endometrial hyperplasia

## 1. Introduction

### 1.1. Endometrium

The uterus (womb) is a pelvic organ with reproductive function, i.e., maintenance of pregnancy. The lower, narrow part, which builds on top of the vaginal opening, was marked as *cervix*, and the broader, upper part, as *corpus*. The *corpus* consists of two types of tissue. The smooth-muscular outer layer (*myometrium*) has the function of expanding during pregnancy, and it follows the development of the fetus. The inner layer (*endometrium*) is subjected to a series of so-called cyclic monthly changes known as the menstrual cycle. The endometrium consists of an outer layer, glandular epithelium, below which is an internal part, stroma. This tissue is hormonally regulated by the steroid hormones estrogens (Es) and progestogens (Ps).

## 2. Gonadotropins and steroid hormones in the reproductive period

The reproductive axis consists of the hypothalamus, pituitary, and ovaries. The gonadotropin-releasing hormone (GnRH) acts on the anterior pituitary by regulating the synthesis and storage of gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). GnRH also regulates the movement of gonadotropins from the reserve pool to a readily released point and their secretion. This action requires pulsatile GnRH release [1]. The secretion of FSH and LH takes place in a coordinated manner so as to regulate the growth of ovarian follicles, ovulation, and the maintenance of the corpus luteum and requires constant pulsatile release of LHRH from the hypothalamus [2]. Both estrogens and progestins help regulate the release of gonadotropins, acting through both the hypothalamus and anterior pituitary. High/low levels of either progestins or a combination of progestins and estrogens, as well as the

length of exposure to these hormones, inhibit/stimulate the release of GnRH, FSH, and LH from the anterior pituitary – a negative/positive feedback control, respectively [3].

In the reproductive age of women, 17 $\beta$ -estradiol (E2) is a major circulating estrogen that is produced by the granulosa cells of the ovary prior to ovulation and by corpus luteum following ovulation. Almost 95 % of circulating Es in premenopausal women consists of 17 $\beta$ -estradiol and the remaining 5 % originates from the peripheral conversion of the estrone (E1) to estradiol [4]. Although a small amount of estrone, the second most important estrogen, is secreted directly from the ovaries and adrenal glands, its main quantity derives from conversion of androstenedione in adipose tissue [5]. The estrogenic potency of estrone is lesser than estradiol, and both, E2 and E1, are biologically equivalent with subtle structural differences and metabolized by the same pathways. Once a woman has reached menopause and ovaries lose their function, estrone becomes a predominant form of estrogen [6]. Studies have shown the trend to higher mortality rate from coronary heart disease in women with lower estrone level, while patients with higher estrone level had lower body weight, less frequent hypertension and diabetes mellitus, and also a lower triglyceride level [7].

During normal ovulatory cycle, the level of E2 varies individually within the range defined for the follicular phase, mid-cycle, and luteal phase. Most of E2 in the circulation is bound to sex hormone-binding globulin (SHBG) and to a lesser extent, to other serum proteins, such as albumin. Only a very small fraction of this hormone is free and is located in the conjugated form [8,9].

During a normal menstrual cycle, E2 secretion is biphasic, and the highest concentration is recorded just prior to ovulation. This growth affects the pituitary gland secretion of FSH and LH by a positive feedback. After ovulation, E2 level rapidly decreases and luteal cells, by their activities, cause mild, subsequent rise and a plateau of E2 during the luteal phase [2]. During pregnancy, the level of E2 in serum increases to much higher values than recorded in the preovulatory peak, and it is maintained during pregnancy [10].

Progesterone (P) belongs to a group of steroid hormones called progestogens, and it is secreted by the *corpus luteum* in the ovary during the second half of the menstrual cycle. During pregnancy, the high levels of progesterone are provided by the secretion of placenta. Contrary to stimulating, proliferative effect of estradiol, progesterone induces secretory activity of the endometrium and has the role of accepting a fertilized egg and beginning pregnancy. In the circulation, progesterone is bound to the corticosteroid-binding globulin and albumin [2,11]. During normal ovulatory cycle, the increase of serum levels of P induces an increase of LH concentration and together with E2, regulates in this way the preovulatory peak of gonadotropins [12]. In vivo studies in humans suggest that P stimulates its own production during the periovulatory and middle luteal period through self-priming [13]. Besides steroids, the ovary secretes peptide hormones (the inhibins) under gonadotropin control. The inhibins belong to transforming growth factors and have the ability to inhibit gonadotropin (FSH) secretion and may also play an important role in ovarian carcinogenesis [14]. The concentrations of inhibin A and inhibin B in circulation

fluctuate during normal menstrual cycle. During the follicular phase, inhibin B is dominant, and during the luteal phase, inhibin A dominates [15].

### 3. Gonadotropins and steroid hormones in menopause

The transition from the reproductive period to the menopause is a gradual process that takes place over many years and is referred to as perimenopause. It starts with the first symptoms of changes in the cyclic occurrence of menstruation and/or bleeding, which may be accompanied by physical and psychological symptoms and ends with the last menstruation. In terms of morphology, this phase is characterized by a sudden drop in the number of primordial follicles in the ovaries, as well as extreme fluctuations in hormone levels [16], so that the frequency of normal ovulatory cycles decreases [2].

It has been shown that some women experience an increase in serum FSH concentrations before the age of 40, especially during the mid-follicular and early luteal phase [17]. Similar increase of FSH was also detected through regular cycles, although there were no clinical manifestations of approaching menopause [18].

Generally, a significant increase in the concentration of FSH is observed approximately 5 years before the onset of menopause and it is positively correlated with age [19,20]. With the onset of menopause, there is an additional increase in FSH levels in serum for about six months, and the peak concentration is detected 3–4 years after menopause. After this period, a slight decline in serum FSH was detected. However, compared with fertile women, levels of gonadotropins remained at elevated levels even 10 years after menopause [19]. Besides FSH, the LH concentration also changes during this period. It has been shown that serum LH increases slightly during 4–5 years of perimenopause in women who still regularly cycled [17]. During the first six months from the onset of menopause, there is an increase in serum concentrations of LH, and the highest level is recorded during the first year of menopause. Over the next 8 years, there is a continuous fall, but as in the case of FSH, the LH level remained elevated compared with fertile period [19]. These data represent the results which should not be generalized and considered as absolute parameters that apply to the period of perimenopause and menopause, since clear markers still have to be identified. In addition, they cannot be reliably interpreted since ovulatory (potentially fertile) cycles can normally take place immediately after the detection of postmenopausal levels of FSH. Both estradiol and inhibin are important regulators of the negative feedback loop of circulating FSH [21–23].

As a consequence of declined follicular function during menopause, the concentration of Es in circulation also decreases. The level of estradiol in the serum of postmenopausal women is less than 15 pg/ml, and the level of estrone is about 30 pg/ml, so that the ratio E1/E2 is 2:1 [11, 24]. The main source of E1, which is the principal form of the postmenopausal estrogen, derives from androstenedione in peripheral adipose tissue and liver [2]. In this period, 95 % of the total synthesis of androstenedione occurs in the adrenal glands and only 5 % in the ovaries [25,26]. Increased conversion of androstenedione to estrone is proportional to the increase of body weight, and it consequently increases the amount of estrogen in the bloodstream [2,26]. The

main source of E2 in postmenopausal women originates from the peripheral conversion of E1. During and after menopause, the concentration of E1 decreases as well as the concentration of E2, so that both forms of estrogen are strongly correlated [27,28]. The concentration of estrone sulfate, which is a metabolite of those estrogens, shows a similar trend of decline in menopausal women. Although it does not belong to the active Es, it can be activated by hydrolysis of the sulfate group [27]. Since premenopause leads to inadequate luteal function or anovulation, progesterone is also lowered in the serum. The level of P is further reduced during the aging process, so it is very low in postmenopausal women [19]. Statistically, approximately 2 % to 3 % of women will develop uterine cancer during lifetime. About 97 % of all uterine cancers originate from endometrial glands and represent endometrial carcinomas [29]. Endometrial carcinoma is the fourth common cancer after breast, bowel, and lung carcinoma [30].

#### 4. Endometrial hyperplasia

Endometrial proliferation is a normal part of the menstrual cycle that occurs during the follicular/estrogen phase of the cycle [31]. If the endometrium is exposed to continuous endogenous or exogenous estrogen in the absence of progesterone, simple proliferation can advance to endometrial hyperplasia, which is the most common precursor of endometrioid adenocarcinoma. Generally, endometrial hyperplasia is the abnormal proliferation of the glands and the stroma characterized by the presence of architectural and cytological changes [32].

#### 5. Classification and histology

As an attempt to correlate morphological features with clinical outcome, the World Health Organization (WHO) classified endometrial hyperplasia:

Nonatypical hyperplasias (typical)
Simple hyperplasia without atypia
Complex hyperplasia without atypia (syn. adenomatous hyperplasia without atypia)
Atypical hyperplasias
Simple atypical hyperplasia
Complex atypical hyperplasia (syn. atypical adenomatous hyperplasia)

**Table 1.** WHO classification of endometrial hyperplasia [33]

The normal proliferative endometrium is characterized by no crowding of glands within the stroma. Morphological features of all endometrial hyperplasia forms include an increase in the gland-stroma ratio, irregularities in gland shape, and variation in gland size. Regardless



of the presence of atypia, simple and complex forms of hyperplasia are distinguished by architectural alterations characterized by glandular complexity and the amount of stroma separating the glands [34]. Hyperplasia generally involves much of the whole endometrium, but sometimes it may be present as a localized lesion and might be associated within an endometrial polyp. Most endometrial hyperplasias are estrogen driven and related to type 1 endometrial carcinoma, the endometrioid endometrial adenocarcinoma [35].

*Simple hyperplasia*, formerly cystic or mild hyperplasia, is a proliferative lesion with minimal glandular complexity and crowding. Histologically, glands are of irregular size from small to those with cystic appearance and shape, separated by abundant stroma. The glandular architectural changes are characterized by varying degrees of irregular branching. Cytologically, the glandular epithelium resembles to proliferative endometrium. It is considered as the least significant form which is not commonly associated with progression to endometrial carcinoma [36].

*Complex hyperplasia*, previously adenomatous hyperplasia or moderate hyperplasia, represents a proliferative lesion with severe glandular complexity and more densely crowded glands. The glands can vary in size and may demonstrate increased structural complexity. Usually, the glands are closely packed, frequently appearing almost back to back and with gland-stroma ratio of more than 2:1 [37,38]. As the severity of hyperplasia increases, the glands become more crowded and more structurally transformed. The complex hyperplasia is considered as the true intraepithelial neoplastic process. Occasionally, this form of hyperplasia may be found coexisting with areas of endometrial carcinoma [39]. Endometrial hyperplasia is further classified based on the presence of cytologic atypia and disordered maturation. Cytologic atypia refers to enlarged epithelial cells that are hyperchromatic with prominent nucleoli, an increased nuclear-to-cytoplasmic ratio, and loss of cellular polarity. Cytologic atypia is the most important prognostic factor for progression to endometrial carcinoma [40].

Thus, the WHO classification also includes lesions termed *simple atypical hyperplasia* and *complex atypical hyperplasia*. Simple atypical hyperplasia is rare, so the term atypical hyperplasia is widely used to refer to all women with simple or complex atypical hyperplasia. The glands in atypical hyperplasia are very closely packed, and endometrial stroma might be seen, separating them [41]. Less than 2 % of hyperplasias without atypia progress to carcinoma, and the mean duration of progression takes almost 10 years. Atypical hyperplasia progresses to carcinoma in 23 % of cases over a mean duration of 4 years [42].

## 6. Endometrial intraepithelial neoplasia system

There is a discussion to replace the WHO classification of type 1 endometrial carcinoma precursors with the endometrial intraepithelial neoplasia classification system. This system was proposed in 2000 by an international group of gynecologic pathologists, and it defines two classes of endometrial changes, endometrial hyperplasia (EH) and endometrial intraepithelial neoplasia (EIN) [43]. In this classification, endometrial hyperplasia refers to changes observed with anovulation or other etiologies of prolonged estrogen exposure. Morphologi-

cally, EH varies from proliferative endometrium with a few cysts to endometria with many dilated glands. This type is also known as cystic glandular hyperplasia, mild hyperplasia, or simple hyperplasia [44]. The term EIN represents monoclonal endometrial preinvasive glandular proliferation as the immediate precursor of endometrial type 1 adenocarcinoma. In EIN, the proliferation of endometrial glands exceeds the stroma (gland/stroma >1) [45]. EIN categories do not correspond directly to the WHO system of classification. Most simple and some complex hyperplasias fall into EH category and many complex hyperplasias with or without atypia are in the EIN category.

## 7. Epidemiology and risk factors

A well-documented study regarding the epidemiology of endometrial hyperplasia included women aged 18 to 90 over the 18-year period. The diagnosis was mostly made in women aged 50–54 years and rarely was found in women under the age of 30. The incidence of simple and complex hyperplasia was 142 and 213 per 100,000 women-years, respectively. The rate of atypical hyperplasia was highest in older women aged 60–64 years, and it was 56 per 100,000 women-years. This rate seems to correlate with age of peak incidence for endometrial cancer [46,47]. Age-specific cancer incidence was demonstrated for the pancreas, bladder, stomach, lung, prostate, ovary, colorectal, and uterine endometrium. One explanation for increased cancer incidence with age is the latency period required for damage to occur and cancer to develop, including the time necessary for accumulation of carcinogen-induced genetic mutations like in oncogenes and tumor suppressor genes but also as a maladaptive response to replicative senescence due to telomere shortening. Also, a deterioration of the innate and the adaptive immune response with aging, referred to as immunosenescence, must be considered [48].

Symptoms of endometrial hyperplasia include heavy or prolonged menstrual periods, intermenstrual bleeding, and prolonged amenorrhea. Postmenopausal women with hyperplasia may experience vaginal bleeding or spotting. However, only minority of women with abnormal uterine bleeding (AUB) are subsequently diagnosed with endometrial hyperplasia [49].

The risk factors for endometrial hyperplasia are the same as for endometrial carcinoma. Most of them include exposure of endometrium to continuous estrogen unopposed by progestin. Unopposed estrogen may be of various sources like early menarche (beginning menstruation before age 12), hormone replacement therapy (HRT) with exogenous estrogen, late menopause (after 52 years of age), estrogen-secreting tumor (some breast cancer types), and nulliparity or low parity. Medical conditions such as diabetes mellitus, polycystic ovary syndrome, or thyroid diseases also increase the risk for hyperplasia and cancer of the uterus. Endometrial hyperplasia is also more likely to occur in women with personal history of breast, colorectal, or ovarian cancer and in women of white race. Endometrial cancer and hyperplasia are more common in Caucasian women, while uterine sarcoma is more common in African American women [50,51].



## 8. Molecular pathogenesis of endometrial hyperplasia and cancer

Although the findings suggest that there are certain molecular characteristics which distinguish types and degrees of endometrial cancer, the molecular mechanisms that underlie the endometrial carcinogenesis are still unclear. Cell changes can begin with genetic aberrations and continue with uncontrolled growth stimulated by tumor promoters.

## 9. Hormone receptors and growth factors

Endometrial tissue is the target tissue for steroid hormones produced by ovaries. Both epithelium and stroma contain receptors for Es and Ps, and ovarian steroids have a fundamental role in the regulation of growth and differentiation of endometrial cells [2]. It seems this influence is partly preserved in well-differentiated tumors of the lower grade, as suggested by data which showed that these tumors are frequently receptor positive than the advanced tumors [52]. Growth factors are, among other influences, regulated by steroid hormones, and they are involved in a paracrine and autocrine regulation of endometrial proliferation. The most often mentioned are the epidermal growth factor (EGF) and transforming growth factor- $\alpha$  (TGF- $\alpha$ ). Both factors are single-chain peptides that exert their effect through the EGF receptor. They were shown to be expressed in normal endometrial tissue [53] and to stimulate growth of cultured endometrial cancer cells [54]. In addition to these two factors, it is considered that the transforming growth factor- $\beta$  (TGF- $\beta$ ) is also involved in the carcinogenesis. This factor is expressed in normal human endometrium and certain endometrial cancer cell lines. In some of these cell lines, like RL95-2, SPEC-2, and KLE, the TGF- $\beta$  inhibits their growth [55]. Among the other growth factors which affect endometrial carcinogenesis, the basic fibroblast growth factor (bFGF) and insulin-like growth factor I (IGF-I) should also be mentioned [56].

## 10. Activation of oncogenes

The most frequently altered oncogenes in endometrial cancer are the point-mutational activation of K-ras. Point mutations of K-ras were found in approximately 10–30 % of endometrial cancers [57]. Also, K-ras mutations have been identified in endometrial hyperplasia and more frequently in complex atypical hyperplasia, suggesting that K-ras mutations may be an early event in endometrial carcinogenesis [58].

In addition to this oncogene, the amplification and overexpressed HER-2/neu (c-erb B-2) was found in about 10–20 % of sporadic endometrial carcinoma cases [59–61]. HER-2/neu gene encodes a membrane receptor protein which is structurally similar to the receptor for epidermal growth factor (EGF-R). In some endometrial carcinomas, the overexpression of oncogenes Myb, Fos, Myc, and fms, as well as their correlation with advanced stages of carcinogenesis and poor prognosis of the outcome of survival, was recorded [57, 62]. Results of some endometrial carcinoma studies detected the overexpression of oncogenes Myb, Fos, Myc, and

fms, as well as their correlation with advanced stages of carcinogenesis and poor prognosis of the outcome of survival [57, 62].

## 11. Inactivation of tumor suppressor genes

Until now, it is observed that mutations in PTEN (phosphatase and tenzin homologue deletion on chromosome 10) tumor suppressor gene, also known as MMAC1 and TEP1, are detected in approximately 50 % of endometrial cancers [63], as well as in 20 % of endometrial hyperplasias, suggesting that these mutations occur relatively early in pathogenesis of this cancer type [64, 65]. PTEN is a dual-specificity protein phosphatase which dephosphorylates tyrosine-, serine- and threonine-phosphorylated proteins. Acting as lipid phosphatase, which is critical for its tumor suppressor function, it removes the phosphate in the D3 position of the inositol ring from phosphatidylinositol 3,4,5-trisphosphate; phosphatidylinositol 3,4-diphosphate; phosphatidylinositol 3-phosphate; and inositol 1,3,4,5-tetrakisphosphate. PTEN is crucial in the control of PI3K-AKT/PKB signaling pathway by dephosphorylating phosphoinositides and thereby modulating cell cycle progression and cell survival [66, 67]. There is a wide spectrum of PTEN mutations in endometrial cancer, which occur in exons 3, 4, 5, 7, and 8 and targeting the phosphatase domain and regions that control the stability and localization of proteins. The consequence of these mutations is reduced or completely absent expression of PTEN [68]. It was shown that progesterone treatment of cultured endometrial stromal cells induces an increase in PTEN levels, while estradiol induces the PTEN phosphorylation. This indicates an outstanding role of PTEN in the development and/or progression of endometrial cancer [69]. Although loss of PTEN function was implicated in the pathogenesis of many different tumors [70], it is believed that the altered expression of PTEN can be a diagnostic marker for the early precancerous conditions of the endometrium [43].

Mutations of the p53 tumor suppressor gene have been found in approximately 10–20 % of all endometrial cancers, with the greatest frequency in the high-grade tumors. Approximately 50 % of grade III tumors type 1 and the rare tumors of type 2 contain mutations in p53, but they have not been reliably detected within the tumor of grade I or hyperplasia [68, 71, 72], so it is considered that they occur in the late stages of endometrial carcinogenesis [56, 68]. The partial role of the p53 in the cell cycle regulation is mediated through the transcriptional activation of other genes, such as p21, followed by inhibition of the cyclin-dependent kinases [73]. Thus, inactivation of p21 could potentially lead to tumor progression. Studies have shown that in approximately 15–40 % of endometrial cancer cases, a loss of p21 gene expression can be detected [74–76]. In addition to p53 and p21, the alterations of p16INK4a (CDKN2A) tumor suppressor gene were also observed. This gene encodes the p16 protein that specifically binds to CDK4 cyclin-dependent kinases, thereby inhibiting the catalytic activity of the CDK4-cyclin D complexes. Until now, it is observed that methylation, mutations, and deletions of p16INK4a gene are rare, and they were detected in approximately 2–6 % of endometrial cancer cases [56, 77], while the loss of expression was found in 20–70 % of cases [78–80].

Endothelins (ETs), ET-1, ET-2, and ET-3, are potent vasoconstricting peptides involved in the pathophysiology of many human malignancies by activating G protein-coupled receptor

(GPCR) subtypes,  $ET_A$  and  $ET_B$  [81]. Expression of ET-1 was detected in normal human endometrium and in endometrial adenocarcinoma. Also,  $ET_A$ R and  $ET_B$ R expression was decreased in endometrial cancer tissue compared with that of normal endometrium [82]. The ET-1-ETRA axis is frequently dysfunctional in numerous types of carcinomas and contributes to the promotion of cell growth and migration [83].

## 12. Microsatellite instability

In addition to mutations of the PTEN gene, microsatellite instability (MSI) is often detected in type 1 of endometrial cancer. MSI was first demonstrated in patients with hereditary nonpolyposis colorectal carcinoma (HNPCC), in which endometrial cancer is often an associated phenomenon. Additional studies have shown that MSI is detected in approximately 25 % of sporadic cases of endometrial cancer [84] or by other studies in 9–45 % of cases [56]. Unlike hereditary forms of nonpolyposis colorectal carcinoma, where subjects with this type of cancer carry mutations of one of the DNA mismatch repair genes, hMLH1, hMSH2, and hMSH6 [85, 86], promoter hypermethylation of the gene hMLH1 represents the predominant cause of MSI only in sporadic cases [87]. There are also data on the hypermethylation of this gene promoter in hyperplasia and in the absence of cancer, which suggests that inactivation of mismatch repair genes precedes the formation of MSI [88].

## 13. Reactive oxygen species

Oxygen may be a source of reactive oxygen species (ROS) due to its incomplete reduction mostly by the oxidoreductase complex I and III of the mitochondrial respiratory chain [89], forming the superoxide anion radical ( $O_2^{\bullet -}$ ). ROS molecules are characterized by a higher reactivity than oxygen in its ground state. The ROS include free radicals (a term that refers to molecules with one unpaired electron in the outer orbital), like superoxide anion radical ( $O_2^{\bullet -}$ ), hydroxyl radical ( $\bullet OH$ ), peroxy radical ( $ROO\bullet$ ), as well as reactive nonradical molecules such as singlet oxygen ( $^1O_2$ ), peroxynitrite ( $ONOO^-$ ), or hydrogen peroxide ( $H_2O_2$ ). Their half-life varies from a few nanoseconds for the most reactive molecules up to a few seconds or hours for stable radicals [90].

There are a few major sources of  $O_2^{\bullet -}$  in the cell: the respiratory chain in mitochondria, endoplasmic reticulum cytochromes (cytochrome P-450-dependent oxygenase, NADPH-cytochrome P-450 reductase), as well as the oxidase contained in the cell cytoplasm and membranes (NADPH oxidase of polymorphonuclear leucocytes, macrophages, and endothelial cells) [91, 92]. The resulting  $O_2^{\bullet -}$  may be converted to  $H_2O_2$  by spontaneous dismutation, as well as by the enzyme superoxide dismutase (SOD). In addition, the  $H_2O_2$  may originate from the monoamine oxidase activity [93] or from the beta-oxidation of fatty acids in peroxisomes [94]. Its reduction is carried out by the enzyme catalase (CAT) and glutathione peroxidase (GPx), which can be considered as the main way of detoxification.  $H_2O_2$  may also be

reduced by the neutrophil myeloperoxidase which catalyzes the conversion of  $\text{H}_2\text{O}_2$  and  $\text{Cl}^-$  to hypochlorous acid ( $\text{HOCl}$ ) and in the presence of transition metals ( $\text{Fe}^{2+}$  or  $\text{Cu}^+$ ), producing  $\bullet\text{OH}$  [95]. The hydroxyl radical is a highly reactive oxidant that reacts almost instantaneously with the surrounding molecules abstracting the hydrogen atom ( $\text{RH}$ ). The resulting free radical ( $\text{R}\bullet$ ) is more stable and therefore has usually longer half-life compared to the  $\bullet\text{OH}$  [96]. Peroxyl radicals have a relatively long half-life, and they are formed in the process of lipid peroxidation, which begins with removal of the hydrogen atom of polyunsaturated fatty acids [97]. Lipid peroxidation in cell membranes can significantly damage their function due to the formation of irreversible disturbance of fluidity and elasticity, which can lead to impairment of cellular homeostasis.

ROS are constantly produced in the body as a result of normal metabolic processes, but there is also a significant influence of external factors. Many chemical and biological agents which are prooxidants under certain conditions can lead to increased production of free radicals. If their production exceeds the capacity of the antioxidant defense, the oxidative stress occurs [96]. ROS can react with any molecules in the cell, thus causing considerable damage which results in cellular dysfunction. These processes are increasingly studied today in the framework of the mechanisms of etiopathogenesis of various diseases. Also, their role in cell signaling, proliferation, differentiation, and programmed cell death – apoptosis – is intensively examined.

## 14. Antioxidant System (AOS)

The term antioxidant refers to a substance that, when present in small amounts compared with the substrate to be oxidized, inhibits or prevents its oxidation. The antioxidant system can be divided into two categories: nonenzymatic antioxidants, which include various compounds of low molecular weight (vitamin E, vitamin C, carotenoids, polyphenols, ubiquinone, and glutathione), and the AO enzyme system [98].

## 15. Nonenzymatic antioxidants

Vitamin E (tocopherol-OH, vitamin E) is a generic name for a group of compounds known as the tocopherols and tocotrienols, and it includes all forms which exhibit biological activity of natural vitamin E (d-alpha-tocopherol) [98]. Vitamin C (ascorbic acid) is the most important hydrophilic antioxidant. Their main function is to prevent peroxidation of lipids in the membrane and, consequently, cell damage. The carotenoids are the vitamin A, which also possess antioxidant properties. Beta-carotene is one of the most studied forms, and its antioxidant function is based on its attribute to quench the singlet oxygen and remove free radicals, thus protecting the cell membrane lipids from oxidative degradation. Polyphenols are a group of compounds with antioxidant capacity to prevent formation of ROS production through inhibition of the enzyme, as well as trace elements, involved in their formation [99].

Ubiquinone prevents lipid peroxidation in liposomes, lipid emulsions, phospholipids, and LDL particles [100]. Glutathione (GSH) is a tripeptide consisting of L-glutamine, L-cysteine, and L-glycine. In addition to its role as a substrate of GSH redox cycles, it also removes the hydroxyl radicals and singlet oxygen and maintains the enzymes and other cellular components in a reduced state [98].

## 16. Antioxidant enzymes

In mammals, three types of SODs have been identified, depending on the cellular localization and prosthetic groups. In the cytoplasm, the predominant form is copper-zinc-superoxide dismutase (CuZnSOD, SOD1), which represents a stable dimeric protein with molecular mass of 32 kDa. It contains copper and zinc in its active site. Copper is considered necessary for the catalytic activity of this enzyme, whereas zinc contributes to its stability [101]. CuZnSOD is also located in the extracellular matrix, and this form is known as the extracellular superoxide dismutase (EC-SOD, SOD3). This form of CuZnSOD is a tetrameric protein with molecular mass of 135 kDa, and it possesses a heparin-binding domain that affects its extracellular distribution [102]. Manganese superoxide dismutase (MnSOD SOD2) is a tetramer enzyme with molecular weight of 88 kDa, containing manganese atom in the active sites and it is located in the mitochondria.

CAT is homo-tetramer enzyme with molecular weight of 240 kDa, with each subunit containing the heme prosthetic group and also the attached NADPH that protects the enzyme from oxidative damage. CAT has a function to decompose  $\text{H}_2\text{O}_2$  to  $\text{O}_2$  and  $\text{H}_2\text{O}$  [103].

GPx family can be divided into two groups: selenium-independent peroxidase presented glutathione S-transferase (GST) and selenium-dependent peroxidases (GPx).

Glutathione S-transferase belongs to the so-called phase II detoxifying enzymes that are involved in conjugation reactions of a wide range of electrophilic xenobiotics (including carcinogens and mutagens). Several selenoprotein glutathione peroxidases are present in human tissues, cell GPx (GPx-1, CGP-x), gastrointestinal GPx (GPx-2, giGPx), plasma (extracellular) GPx (GPx-3, eGPx), and phospholipid hydroperoxide GPx (GPx-4, PHGPx) and GPx-6, which is only expressed in the epithelium of the olfactory system [104]. With the exception of PHGPx which is a monomer (19 kDa), other forms of GPx are composed of four identical subunits of a molecular weight of 19–25 kDa. Each subunit in its active site contains a selenocysteine ( $\text{Cys}^{\text{Se}}$ ). The enzyme uses a reduced GSH as a source of reducing equivalents (electrons) to regenerate  $\text{Cys}^{\text{Se}}$  to the reduced state [105]. Glutathione reductase (GR) is an enzyme that catalyzes the reduction of oxidized glutathione GSSG to GSH and it is essential for the GSH redox cycle [106].

## 17. Oxidative stress and cell signaling

Because of their high reactivity, elevated ROS concentrations represent a great danger for biomolecules. At physiological concentrations, these molecules are often necessary for normal



functioning of cells as second messengers in the transduction of the cell signaling [107]. They can be activated in such a way as to prevent or potentiate the cell death. Many signaling pathways in the cell can be activated in both directions (cell survival or apoptosis), which depends on the type and duration of oxidative stress or cell types. Also, some of these pathways can affect the activation or suppression of other signaling pathways in the cell.

It is difficult to determine which type of ROS activates signaling pathways, because of their extremely rapid conversion to other forms or due to the conversion of acid conjugates or complexes with transition metals [108]. It is believed that  $H_2O_2$  is highly suitable as a secondary messenger because it does not randomly react with all of the molecules like other forms of ROS, but tends to oxidize the -SH group of cysteine (Cys), which is then reduced by GSH [109].

In this way, by redox cycling of Cys, many transcription factors are regulated, such as activating protein 1 (AP-1) [110], nuclear factor NF-IL6 [111], and proteins important in cell signaling and cancerogenesis: protein kinase C (PKC),  $Ca^{2+}$ -ATPase, collagenases, and SRC tyrosine kinase [108]. It is known that ROS are critical molecules in regulation not only of the AP-1 but also AP-2 [112] and of nuclear factor NF-kappaB [113] transcription families, which have a decisive role in cell proliferation, differentiation, and morphogenesis.

Other processes induced by hydrogen peroxide included activation of the stress-activated protein kinase/c-Jun N-terminal kinases (SAPK/JNK), the increased c-Jun phosphorylation, activation of caspase 3 (CPP32), and decomposition of poly(ADP-ribose) polymerase (PARP), which are associated with the apoptosis process [114]. Besides regulating the activity of cell proteins,  $H_2O_2$  also induces the expression of many genes [115]. In addition, these molecules are responsible for the disruption of cell signaling and regular patterns of gene expression [116], which can lead to a number of pathological processes including carcinogenesis. The process of carcinogenesis is complex and consists of a series of changes at the cellular and molecular levels and in at least three stages: initiation, promotion, and malignant conversion, i.e., progression [117].

## 18. AO antioxidant status and carcinogenesis

In relation to carcinogenesis, it is known that the AO system has a role in preventing its occurrence and promotion. The studies AO status in tumor tissues have not yet yielded results that could lead to general conclusions about AO defense in tumor tissues. Since the carcinogenesis occurs in several stages, it is likely that the antioxidant defense depends on the type of cell and tissue [118]. Mammalian cells and tissues differ significantly in the generation of ROS. They also vary in antioxidant activity, induction capability, and cell repair capacities which altogether results in a different susceptibility of mammalian tissues for tumor induction [119-121].

Our earlier studies indicated a significant role of oxidative-induced injury in the breast carcinogenesis, particularly during the later stages of aging [122]. It was also observed that chemotherapy and radiotherapy promote further oxidative shift, which potentiates already

existing chronic oxidative stress linked to breast cancer [123]. It is believed that the high antioxidant capacity protects DNA from oxidative damage and mutagenesis but also can protect the cells in the stage of initiation of increased oxidative toxicity, thus favoring their clonal expansion and tumor progression [124]. It has long been known that oxidizing agents may be cytotoxic, although under certain circumstances, can promote cell growth and facilitate the clonal expansion of the initiated cells in carcinogenesis [125].

## 19. AO enzymes in gynecologic disorders

Some previous studies have shown that compared to healthy people, women with benign and malignant changes in the genital tract have increased level of lipid peroxidation and altered activity of AO enzymes in peripheral blood and tissue. Chiou and Hu [126] have detected that the activity of SOD in plasma and erythrocytes of patients with cervicitis and uterine myoma was lower compared to that of healthy women. At the same time, patients with cervicitis had an increased level of CAT and GPx activity, while their activity in patients with uterine fibroids (leiomyoma) was reduced. Similar results regarding the activities of SOD, CAT, and GPx in erythrocytes of patients with cervicitis were obtained by Manoharan et al. [127]. These authors also found that the activity of these enzymes was lower in patients with cervical cancer. Research of Kolanjiappan et al. [128] and Manoharan et al. [127] showed that the level of lipid peroxidation increased and the concentration of the antioxidant GSH, vitamin E, and CAT decreased in erythrocytes of patients with cervical cancer. These patients had altered activity of Na<sup>+</sup>K<sup>+</sup>-ATPase in erythrocytes compared to healthy persons. Our previous results showed that AO status in blood of gynecological patients varies with diagnosis and the enzyme type. Generally, both reduction in antioxidants and elevation of lipid peroxidation were observed. Lipid hydroperoxide level was negatively correlated to SOD and GPx activities and concurrently positively correlated with CAT activity. In addition, the lipid hydroperoxides/glutathione peroxidase ratio increased, according to the type of uterine disorder [129-131]. The perturbation of antioxidant status was more pronounced in blood of patients with hyperplastic and adenocarcinoma lesions compared to those with benign uterine changes such as polypus and myoma. Our results of AO status in endometrial tissue showed significant decrease of SOD activity in women with hyperplasia and adenocarcinoma. In both types of hyperplasia, activities of GPx and GR were increased to 60 % and 100 % on average, while in adenocarcinoma patients, only GR activity was elevated to 100 %. CAT activity was significantly decreased in adenocarcinoma patients (47 %). Lipid hydroperoxides level was negatively correlated to SOD and CAT activities and positively correlated to GPx and GR activities [132]. Since association of different clinical risk factors and various types of gynecologic pathologies is still not fully known as well as their influence on AO status, in our latest study, we evaluated the influence of diagnostic categories, age, and reproductive factors on AO status in blood of gynecological patients [133]. The obtained results showed that reproductive and other factors may be associated, at least partially, with AO capacity and ability to defend against the oxidative damage in gynecological patients.

The AO status and hormone influence were studied during the menstrual cycle and postmenopause in healthy women and those with gynecologic disorders. The SOD was found to have a role in maintaining luteal cell integrity and steroidogenic capacity in fertile women [134]. An increase in the GPx activity was observed during the menstrual cycle, from the late follicular to the early luteal phase. The rise in GPx activity is related to increased ovarian production of estrogen that occurs in that particular period of menstrual cycle [135]. Decrease in GPx activity has been noted in the endometrium and blood in late-menopausal women [136]. Menopause is accompanied by hormone imbalance. A significant fall of the estrogen serum level with rise of follicle-stimulating hormone (FSH) has been recorded in postmenopausal women compared to premenopausal women [137]. Hormone replacement therapy (HRT) shows protective antioxidant role by reduction of lipid peroxide (LOOH) serum levels [138]. It is also found that HRT positively correlates with SOD activity in postmenopausal women [139].

We have shown that AO enzyme activity and lipid hydroperoxide level in patients with endometrial polyps are influenced by the changes in sex hormones during the menstrual cycle and in menopause [140]. In this study, we aimed to examine the AO status in menstrual cycle and postmenopause of women with endometrial hyperplasia simplex as well as the relationship between sex hormones and AO parameters.

## 20. Methods

**Subjects.** The material used in this study consisted of 35 blood and tissue specimens of women admitted to the Department of Gynecology and Obstetrics for gynecological evaluation within routine checkups or for abnormal uterine bleeding (prolonged menstrual bleeding and postmenopausal bleeding). On the basis of diagnosis and histological examination, subjects were diagnosed with *hyperplasia simplex endometrii*, and the specimens were taken after obtaining the informed consent. The study was conducted prospectively and it was approved by the Human Studies Ethics Committee of the Clinical Center. The protocol was consistent with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects). None of them had undergone hormone therapy or any other medical treatment in the last six months. Patients were divided as follows: 10 in the proliferative (follicular phase, F) (age, 40–52 years; median 46 years), 15 in the secretory (luteal phase, L) (age, 27–53 years; median 44 years), and 10 in the postmenopause (PM) (age, 47–60 years; median 53 years).

**Samples.** Samples were collected and prepared for enzyme assays according to the procedures described previously [129,131]:

Venous blood samples were collected into heparinized tubes on the same day of uterine biopsy and aliquoted immediately. For SOD assay (OxisResearch™), blood was centrifuged at 2500 g for 5 min. Plasma was discarded and pellet was resuspended in 4 packed-cell volume of ice-cold demineralized ultrapure water (MilliQ reagent grade water system, Millipore Corp., Bedford, MA, USA). After addition of ethanol/chloroform extraction reagent (62.5/37.5 vol/vol) to remove hemoglobin interference, samples were centrifuged at 3000 g for 10 min

(Eppendorf centrifuge 5417, Eppendorf AG, Hamburg, Germany). Upper aqueous layer was collected and kept at  $-70^{\circ}\text{C}$  until assay.

Fresh endometrial tissue samples were washed in saline solution and homogenized in phosphate buffer containing  $0.05\text{M KH}_2\text{PO}_4$  and  $1\text{ mM EDTA}$ , pH 7.8 (1 g tissue per 2 ml buffer) in a Teflon/glass homogenizer (Spindler & Hoyer, Göttingen, Germany) and frozen at  $-70^{\circ}\text{C}$  for 20 h in order to disrupt cell membranes. For SOD assay (OxisResearch™), thawed homogenates were vortexed 1 min and centrifuged at 8600 g, for 20 min at  $4^{\circ}\text{C}$  (Eppendorf centrifuge 5417, Eppendorf AG, Hamburg, Germany). According to manufacturer's recommendation, after addition of ethanol/chloroform extraction reagent (62.5/37.5 vol/vol) to completely remove hemoglobin interference, samples were centrifuged at 6000 g for 20 min, at  $4^{\circ}\text{C}$  (Beckman centrifuge J2-21, Beckman Instruments Inc., Palo Alto, CA, USA). Upper aqueous layer was collected and kept at  $-70^{\circ}\text{C}$  until assay. The enzyme activities and lipid hydroperoxide (LOOH) concentration were monitored spectrophotometrically (Perkin Elmer Spectrophotometer, Lambda 25, Perkin Elmer Instruments, Norwalk, CT, USA).

The specific enzyme activities were expressed as Units (U) or mU per milligram of total cell protein (U or mU/mg protein), and LOOH concentration was expressed as nmol/mg protein. Protein concentration in tissue homogenates was performed by the method of Lowry et al. [141] and expressed as mg/ml. Plasma follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E), and progesterone (P) levels were analyzed using standard radioimmunoassay (RIA) methods by the hormone analysis laboratory.

*Enzyme Assays.* Enzyme assays were performed as described previously [132]:

*Assay of SOD activity.* Determination of SOD activity was performed using Oxis Bioxytech® SOD-525™ Assay (Oxis International, Inc., Portland, OR, USA). The method is based on SOD-mediated increase of autooxidation of 5,6,6a11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. The SOD activity is determined from the ratio of the autooxidation rates in the presence ( $V_s$ ) and in the absence ( $V_c$ ) of SOD. One SOD-525 activity unit is defined as the activity that doubles the autooxidation rate of the control blank.

*Assay of CAT activity.* CAT activity was determined by the method of Beutler [142]. The reaction is based on the rate of  $\text{H}_2\text{O}_2$  degradation by catalase contained in the examined samples. The reaction was performed in an incubation mixture containing 1 M Tris-HCl, 5 mM EDTA, pH 8.0, and monitored spectrophotometrically at 230 nm. One unit of CAT activity is defined as 1  $\mu\text{mol}$  of  $\text{H}_2\text{O}_2$  decomposed per minute under the assay conditions.

*Assay of GPX activity.* GPx activity was assessed using the Oxis Bioxytech® GPx-340™ Assay (Oxis International, Inc., Portland, OR, USA), based on the principle that oxidized glutathione (GSSG) produced upon reduction of an organic peroxide by GPx, is immediately recycled to its reduced form (GSH) with concomitant oxidation of NADPH to  $\text{NADP}^+$ . The oxidation of NADPH was monitored spectrophotometrically as a decrease in absorbance at 340 nm. One GPx-340 unit is defined as 1  $\mu\text{mol}$  of NADH oxidized per minute under the assay conditions.

*Assay of GR activity.* Activity of GR was measured using the Oxis Bioxytech® GR-340™ Assay (Oxis International, Inc., Portland, OR, USA). Assay is based on the oxidation of NADPH to



NADP<sup>+</sup> during the reduction of oxidized glutathione (GSSG), catalyzed by a limiting concentration of glutathione reductase. The oxidation of NADPH was monitored spectrophotometrically as a decrease in absorbance at 340 nm. One GR-340 unit is defined as 1  $\mu$ mol of NADH oxidized per minute under the assay conditions.

**Lipid hydroperoxides.** Concentration of LOOH was measured by Oxis Bioxytech® LPO-560™ Assay (Oxis International, Inc., Portland, OR, USA), which is based on the oxidation of ferrous (Fe<sup>2+</sup>) ions to ferric (Fe<sup>3+</sup>) ions by hydroperoxides under acidic conditions. Ferric ions then bind with the indicator dye, xlenol orange, and form a colored complex. The absorbance of the complex was measured at 560 nm. Since hydrogen peroxide content in many biological samples is much higher than that of other hydroperoxides, samples were pretreated with catalase to decompose the existing H<sub>2</sub>O<sub>2</sub> and eliminate the interference.

**Statistics.** Statistical analysis was carried out by the use of the Kruskal-Wallis test followed by the Dunn's *post hoc* test, which considered the unequal and small sample sizes we used in this study. A linear regression model was used to evaluate associations between hormonal and antioxidant variables. Before plotting the data in the regression study, the normality test on the variables was performed, and the values of estradiol and progesterone were log transformed. The 95 % confidence intervals (CIs) for the regression lines were calculated. Two-tailed *p* values are given throughout. All data were analyzed using GraphPad Prism software.

## 21. Results

The phase-related concentrations of gonadotropins and sex hormones are reported in Table 2. Significant changes were observed in FSH (H=12.75, *p*<0.01, Kruskal-Wallis), LH (H=8.98, *p*<0.01, Kruskal-Wallis), and estradiol (H=7.93, *p*<0.05, Kruskal-Wallis) concentrations.

	Follicular phase	Luteal phase	Postmenopause
FSH (U/L)**	14.30±3.51	13.20±2.21	38.88±7.88
Median	11.50	10.80	32.15
(Min/max)	(7.50–31.50)	(0.1–31.50)	(14.30–75.00)
LH (U/L)**	3.33±1.70	3.93±0.96	11.88±2.47
Median	2.50	3.10	11.25
(Min/max)	(0.60–9.40)	(0.60–11.20)	(1.30–24.00)
Estradiol (pg/ml)*	39.30±7.59	71.41±14.07	5.16±1.16
Median	48.80	56.50	3.80
(Min/max)	(12.60–57.20)	(10.00–208.10)	(0.70–11.40)
Progesterone (nmol/L)	7.40±1.09	8.83±2.68	5.16±1.16
Median	6.30	5.30	3.80
(Min/max)	(5.20–12.10)	(1.30–41.60)	(0.70–11.40)

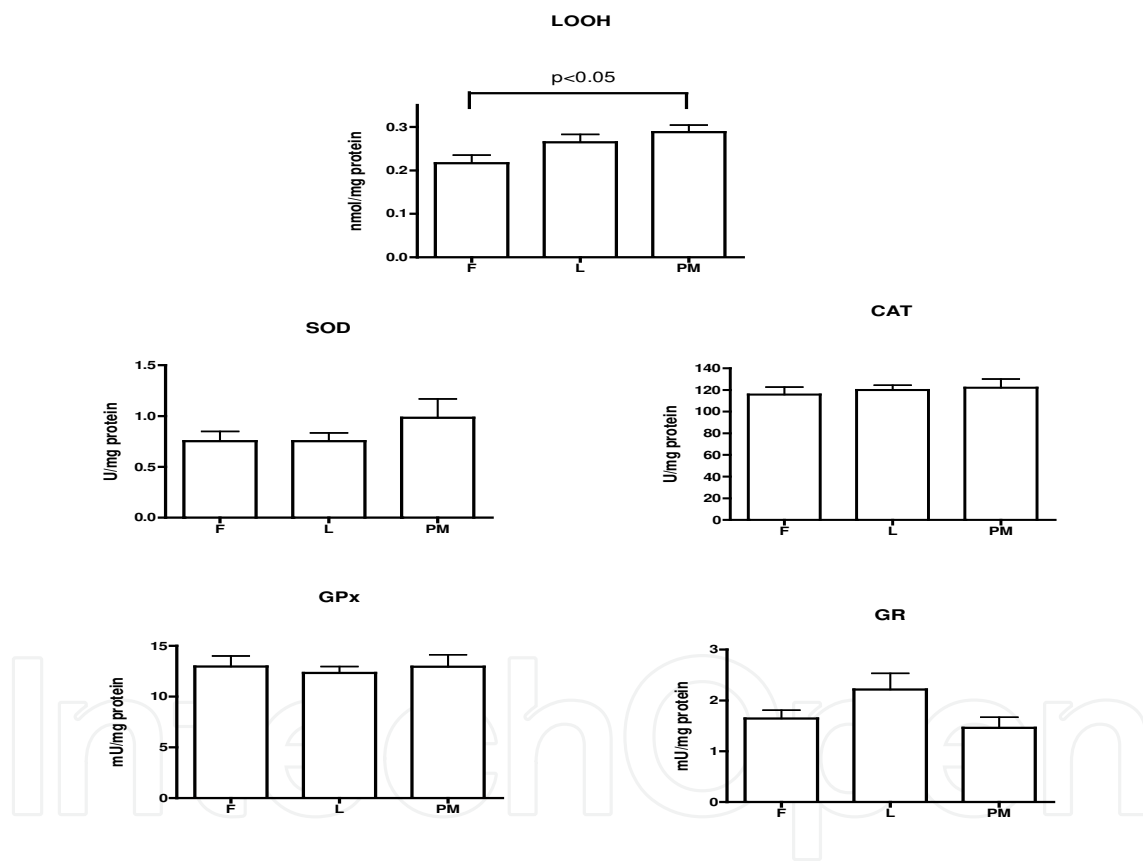
**Table 2.** Changes in hormone levels during follicular phase, luteal phase, and in postmenopause (data are expressed as mean ± SEM; \* *p*<0.05, \*\**p*<0.01)



21.1. Antioxidant parameters and correlation with sex hormones in blood

Figure 1 shows the phase-related changes of LOOH concentrations and AO enzyme activities in the blood of examined patients. The significant change with respect to the phase was observed in LOOH concentrations ( $H=5.76$ ,  $p<0.05$ , Kruskal-Wallis). In the follicular phase, it was significantly lower than in the postmenopause ( $p<0.05$ , Dunn test). There were no significant changes of AO enzymes in the examined phases.

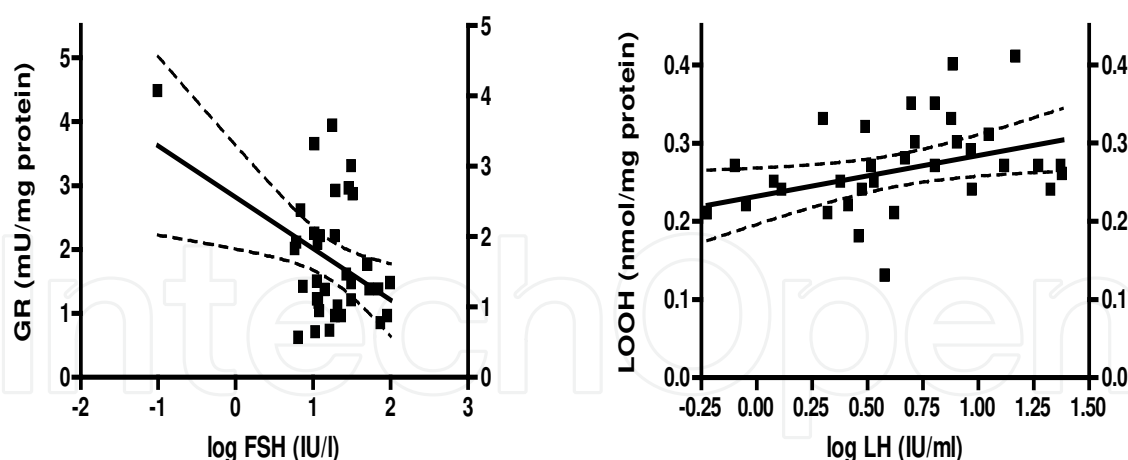
The linear regression analysis of individual hormonal variables against antioxidant parameters in blood (Figure 2) showed a significant negative correlation between FSH concentrations and GR activity ( $r=-0.42$ ,  $p<0.05$ ), as well as a significant positive correlation between LH and LOOH concentrations ( $r=0.38$ ,  $p<0.05$ ). No significant correlations were found between other hormones and antioxidant variables.



**Figure 1.** Changes in blood LOOH concentrations and AO enzyme activities in follicular phase (F), luteal phase (L), and postmenopause (PM) in blood of patients with hyperplasia simplex. Data are shown as mean  $\pm$  SEM.  $P$  values refer to the results of the Dunn test

21.2. Antioxidant parameters and correlation with sex hormones in hyperplasia simplex tissue

The phase-related changes of LOOH concentrations and AO enzyme activities in hyperplasia simplex tissue are shown in Figure 3. The LOOH concentration significantly differed with



**Figure 2.** Linear regression line and 95 % CI to study the relationship between log FSH and GR activity; log LH and LOOH concentration in the blood of patients with hyperplasia simplex

respect to the phase ( $H=7.74$ ,  $p<0.05$ , Kruskal-Wallis), and it was significantly elevated in luteal phase and in postmenopause, in comparison to the follicular phase ( $p<0.05$ , Dunn test).

Unlike blood, where no changes in AO enzyme activities were recorded, we found significant phase-related changes of SOD ( $H=9.11$ ,  $p=0.01$ , Kruskal-Wallis) and CAT activity  $H=7.60$ ,  $p<0.05$ , Kruskal-Wallis). Both enzymes had similar activity pattern, which was higher in luteal phase and in postmenopause, compared to follicular phase ( $p<0.05$ , Dunn test). The phase-related activity of GPx and GR did not show any statistical difference.

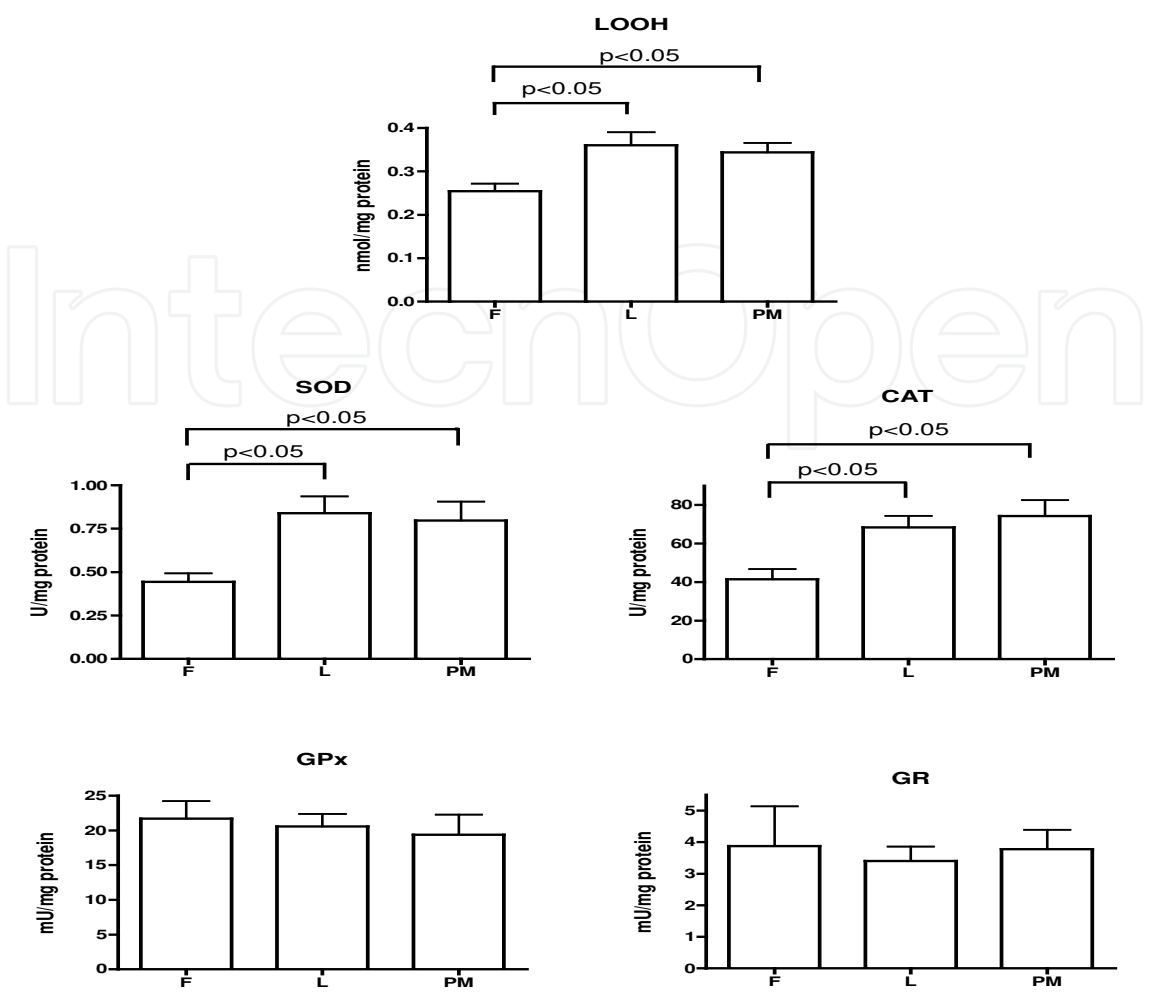
The linear regression analysis of hormone levels on the examined AO parameters in hyperplasia simplex tissue showed a negative correlation between progesterone and GR activity (Figure 4) ( $r=-0.36$ ,  $p<0.05$ ).

## 22. Discussion

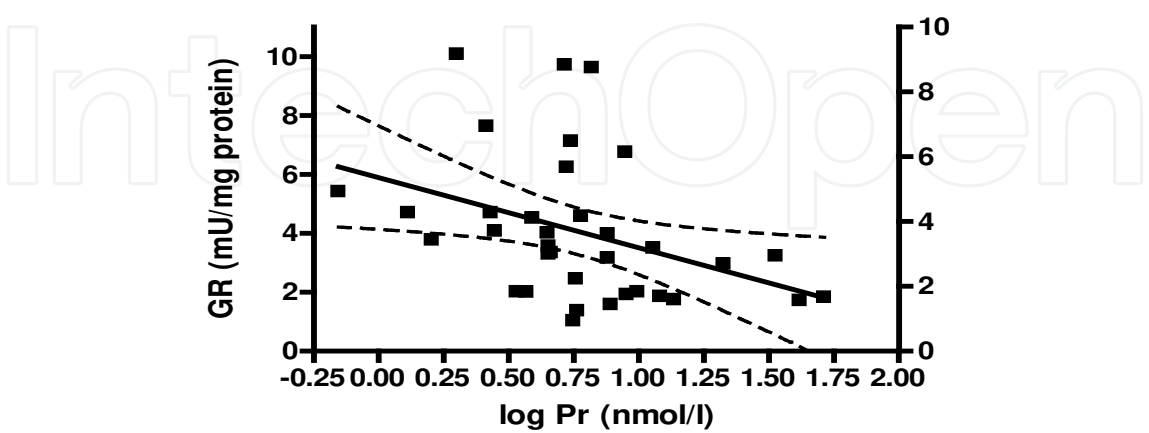
Studies have shown a different AO status and sex hormone influence during menstrual cycle and postmenopause in healthy women and those with ovarian disorders [143-146], but we found no data regarding that relation in patients with endometrial hyperplasia simplex.

In the blood of these patients, we detected a lower level of LOOH in the F phase in comparison to the postmenopause. In hyperplastic tissue, LOOH level was lower in the F phase than in L phase and postmenopause. The activities of SOD and CAT were also lower in F phase when compared to the L phase and postmenopause. There was a negative correlation between FSH/P concentrations and GR activity in the blood and hyperplastic tissue, respectively. Positive correlation between LH and LOOH concentrations was recorded in the blood.

Similar pattern of LOOH concentration and SOD activity in endometrium of healthy women throughout the menstrual cycle was also observed in [134]. They found that LOOH concentration increased from early proliferative phase to mid-late proliferative phase and further



**Figure 3.** Changes in endometrial LOOH concentrations and AO enzyme activities in follicular phase (F), luteal phase (L), and postmenopause (PM) in hyperplasia simplex tissue. Data are shown as mean  $\pm$  SEM. *P* values refer to the results of the Dunn test



**Figure 4.** Linear regression line and 95 % CI to study the relationship between log Pr and GR activity in hyperplasia simplex tissue.

increased in the late secretory phase. The SOD activity increased from early proliferative phase to mid-late proliferative phase, further increased in the mid-secretory phase, and then decreased in the late secretory phase. Previous investigations of immunohistochemical distribution of SOD in human endometrium during menstrual cycle also showed that surface and glandular epithelia contain SOD during proliferative and secretory phases except just prior to the menstruation [147].

The study of Ota et al. [148] regarding SOD expression in endometrium during the menstrual cycle of healthy fertile women and women with diagnosed endometriosis and adenomyosis have shown the phase-dependent changes of SODs in glandular and surface epithelia in healthy women. Specifically, the expression of copper, zinc SOD was lowest during the early and mid-proliferative phases and then gradually increased and was most pronounced in the early and mid-secretory phases. The expression of manganese SOD reached a peak in the late secretory phase. In women with endometriosis and adenomyosis, the expression of both SODs was constantly elevated compared to healthy women throughout the menstrual cycle, which suggested a key role of superoxide in infertility caused by endometriosis and adenomyosis [148]. Our recent findings in women with endometrial polyp showed the opposite pattern of LOOH concentration and SOD activity in blood and polyp tissue than in women with hyperplasia simplex. Both parameters were higher in the proliferative phase compared to the secretory phase or postmenopause in blood and endometrium of the examined women [140].

Regarding CAT, in [149], it was found that CAT expression in healthy women fluctuated greatly during the menstrual cycle and the surface epithelium showed a similar pattern to that in the glandular epithelium. The expression was the lowest during the early proliferative phase, increased during the mid-proliferative phase, and peaked in the late secretory phase. In patients with endometriosis, the CAT expression did not fluctuate during the cycle, but it was consistently elevated throughout the menstrual cycle when compared to healthy women. Likewise, in women with adenomyosis, the CAT expression did not vary during the cycle in comparison to healthy ones, and it was significantly higher than in patients with endometriosis [149]. In women with endometrial polyp, we found no significant change of CAT activity in different phases [140]. In this study, however, the CAT activity in endometrium of patients with simple hyperplasia was also similar to the healthy women.

Studies in women with gynecologic disorders indicate a different AO status, as one of the possible factors contributing to the development of oxidative stress [150]. There are also studies which investigated the role of oxidative stress and hormones in development of gynecologic pathologies. For example, in [151], it was found that FSH, LH, and estrogen could induce ROS production at different levels in ovarian epithelial carcinoma and may therefore participate in cancer development process. FSH was found to increase cell proliferation in ovarian epithelial carcinoma (OEC) [152], and LH may also be involved in OEC development under pathological conditions [151].

Simple hyperplasia is the most common type of endometrial hyperplasia and the type most likely to spontaneously regress, and it rarely progresses to endometrial cancer [42, 51, 153]. The LOOH concentrations and AO enzyme activities in this study which were similar to

the healthy women point to the preserved cellular AO status in these patients. Endometrial hyperplasias are generally considered as precancerous lesions and are treated either conservatively or surgically. The regression of hyperplastic to normal endometrium is the main purpose of any conservative treatment. It is based on the administration of agents, like progestogens [154], which have an indirect antiestrogenic action and also a direct antiproliferative effect on the endometrium [155]. Also, therapeutic application of gonadotropin-releasing hormone analogue (GnRHa) in women with hyperplasia was associated with high regression rates. The regression to normal endometrium is considered to be due to decreased gonadotropin levels as a result of pituitary downregulation or to the decreased ovarian steroidogenesis following low gonadotropin levels [156, 157]. The results of this study also showed that gonadotropins and progesterone influenced oxidant/antioxidant parameters in hyperplastic patients. Although we found no significant changes of GR activity among the menstrual cycle phases, FSH/P was negatively correlated with GR activity in the blood and hyperplastic tissue, while positive correlation between LH and LOOH concentrations was recorded in the blood. Our previous study in women with endometrial polyp also showed the influence of gonadotropins on AO status. In these patients, we observed a negative correlation between FSH/LH and GPx activity and also between LH and SOD activity [140].

The role of gonadotropins in gynecological diseases is not fully clarified. In ovarian epithelial cancer (OEC), gonadotropin theory proposes that elevated serum FSH and LH levels contribute significantly to its development [158]. FSH generally acts through its membrane-bound receptor which activates the intracellular signaling cascade, starting with cyclic AMP/protein kinase A (cAMP/PKA) that is followed by phosphorylation of specific transcriptional factors, like cAMP-response element-binding protein (CRE), or p38 MAPK, which controls other kinase cascades. The FSH receptor can also activate extracellular signal-regulated protein kinases (ERK-s) [159].

It was shown that synthesis of antioxidants, such as glutathione in the ovary, is regulated by gonadotropins, but exact mechanisms are still unknown [160]. One of the mechanisms behind FSH and antioxidants interaction is through activation of transcriptional factors, like Nrf2. The induced Nrf2 binds to the antioxidant-response element (ARE), thus coordinately regulates the expression of AO genes [161].

The pathogenesis of endometrial hyperplasia is still not fully understood. Prolonged estrogen stimulation is considered as one of the factors related to the etiology. This study showed that patients with endometrial hyperplasia simplex have similar AO status like healthy women, and it also demonstrated the relation of hormones and prooxidant/antioxidant parameters in this gynecologic disorder. Since simple hyperplasia may spontaneously regress, these results point to the preserved AO capacity as a potentially important factor in the regression mechanisms. However, the role of ROS production as a risk factor for endometrial hyperplasia still needs to be clarified as well as the role of AO status in response to gonadotropins and sex steroids.



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